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Genetic identification of fungi colonising seedlings of the Scots pine (*Pinus sylvestris* L.) in the forest nursery in Korenevka (Belarus)

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Abstract

DNA amplification was investigated in order to determine fungal species present in the Koronevka forest nursery (eastern part of Belarus). For this purpose, needles and roots of Scots pine (*Pinus sylvestris* L.) seedlings as well as soil collected around roots were examined for ITS1–5.8S RNA-ITS2 region sequences and compared with GenBank data. DNA analysis of seedlings microflora and soil samples allowed identification of twelve different species of fungi. Among these *Cladosporium herbarum* Link, *Davidiella tassiana* Crous and U. Braun, *Alternaria alternata* Nees and *Cryptococcus pinus* Vuill. were often found in symptomatic needles. Pathogenic fungal species were detected in 57% of shrunken needles. Examination of DNA extracted from seedling roots revealed occurrence of *Wilcoxina mikolae* Chin S. Yang and Korf, *C. herbarum*, and *A. alternata*. In soil samples there were identified fungi of the same species, with predominance of mycorrhizal fungus *W. mikolae* (in 100% of samples) and *C. pinus* (in 20% of samples). The results demonstrated usefulness of molecular markers for the detection and identification of fungi.

Key words

ITS markers, molecular identification, pathogenic fungi, micorrhizal fungi, *Pinus sylvestris*

Introduction

At the present time, the production of plants in nurseries is the main source of seedlings for forest silviculture. Basic forest management rules which affect quality and quantity of seedlings comprise following sanitary requirements and implementing complex measures against pathogens, pests and negative environmental influences (Orlikowski *et al.* 2007). Assessment of the threat of plant infection as well as detection and identification of pests and pathogens in nurseries are important aims of phytopathological monitoring.

Development of mol–ecular biology resulted in improvement of genome and proteome analysis that allowed to develop a new branch of phytopathology – molecular phytopathology. The main directions of

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this branch are: molecular mechanisms of host-parasite interactions, genetic and biochemical basis of pathogenicity, origin and evolution of gene of virulence, development of methods of early diagnostics and taxonomical identification of pathogens (Dyakov *et al.* 2007).

The majority of molecular phytopathology methods have been based on the detection and analysis of genetic material (DNA). The objects for DNA isolation may constitute different fractions of damaged plants (including dead parts) as well as water soil. Generally, a small quantity of samples is sufficient for molecular analysis (a few milligrams of plant tissue; a few grams or ml of soil and water, respectively). Modern DNA assays allow to detect fungus infection even when a sample contains only one living cell of a pathogen.

Molecular monitoring in forest pathology is particularly relevant in forest nurseries, and DNA-based markers can be used both for direct analyses of seedling infections and for prevention activities such as *e.g.* examination of potential sources of infection (soil, water, *etc*.). Previous studies demonstrated usefulness of molecular markers such as RAPD or ISSR in identification of species based on DNA isolated from pure cultures (Oszako *et al.* 2007).

The aim of this study was to identify the spectrum of pathogenic fungi in seedlings of Scots pine (*Pinus sylvestris* L.) and soil sampled from forest nursery with the use of DNA assays.

Materials and methods

Samples were collected during the 1st decade of May 2009, in the forest nursery of the Forest Experimental Station in Korenevka (Forest Institute of National Academy of Sciences of Belarus). That period was characterised by long-lasting lack of rain causing massive withering of Scots pine seedlings.

During the experiment, samples for each seedling were collected from living plant parts (damaged shrunken needles) and from parts of roots including soil from root zones.

Needle and root samples were rinsed out with sterile distilled water before the analyses. DNA from each sample was isolated separately according to the procedures designed for plants and soil (Padutov *et al.* 2006). PCR-analysis was carried out with Dream Taq™ Green PCR Master mix (Fermentas). The primers for amplification were ITS1 and ITS4 (White et al. 1990). Electrophoresis was performed in 2% high efficiency separation agarose (Pharmacia Biotech.). For identification, DNA bands were cut and sequenced on ABI Prism 310 (Applied Biosystems) automatic sequencer with the use of BigDye Terminator Sequence Kit v.3.1. Obtained sequence data were analysed in comparison with the data available in NCBI GenBank (www.ncbi.nlm.nih.gov.).

Results and discussion

The results of DNA analyses of seedling and soil microflora indicated 12 different fungal species. Primary species diagnostics was based on amplicon size of 18SRNA-ITS1–5,8RNA-ITS2–28SRNA region. The length of this loci of ribosomal DNA is practically constant at a species level, and to a certain extent this loci can be used as a primary diagnostic feature.

The genetic material in damaged needles as well as in collected roots and soil contained more than one micromycetes species at the same time. The quantitative content of various species of fungi in the samples was uneven which resulted in differentiated colour intensity of amplicons. For sequencing and further species identification amplicons of these species were used (Fig. 1).

The seedling analyses revealed 5 species of fungi which infected seedling needles. It is worth pointing out that the presence of fungal DNA was found only in damaged shrunken needles. DNA of pathogens was not detected in living needles. The number of fungi species in infected needles ranged from 1 to 4 depending on samples tested. In PCR-spectrum most often found species were: *Cladosporium herbarum* Link and *Davidiella tassiana* Crous and U. Braun – with the fragment of 553 base-pairs (bp) present in 85% samples *Alternaria alternata* Nees – 569 bp in 43% samples, *Cryptococcus pinus* Vuill. – 641 bp in 14% samples.

Unknown fungi characterised by amplicon of 630 bp, were found in 57% of samples from damaged needles. Unfortunately, examination of NCBI GenBank data did not show even closely related fungi and that is

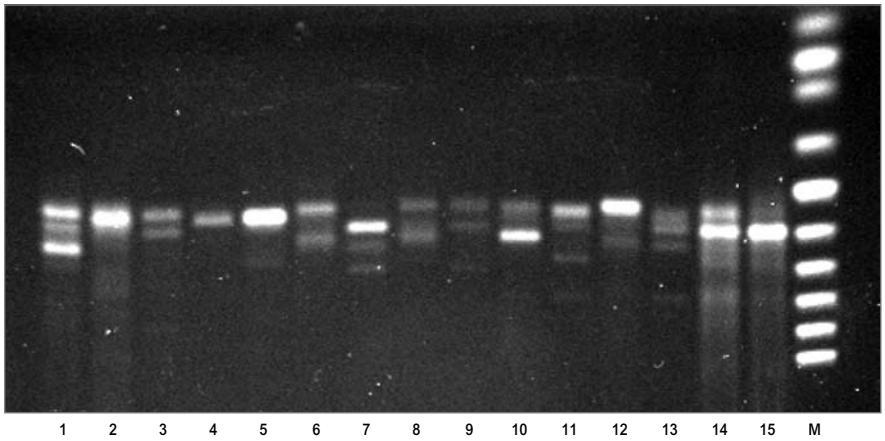

Fig. 1. PCR-spectrum with ITS1/4 primers obtained for fungal species from the forest nursery in Korenevka. Lanes: 1, 2, 4, 6, 8, 10, 12 – damaged needles; 3, 5, 7, 9, 11, 13 – roots; 14, 15 – soil; M – molecular weight marker

why they were deposited as GQ413953 in NCBI Genbank for later isolation.

Examination of seedling roots showed presence of fungal DNA in more than 90% sampled cases. 70% of this DNA constituted genetic material of mycorrhizal fungi *Wilcoxina mikolae* Chin S. Yang and Korf, 45% to *C. herbarum*, and 30% to *A. alternata*. Common species *C. herbarum* and *A. alternata* (often recognised as pathogens of seedlings) were detected even in cases when obvious signs of shrinkage of seedlings was not observed. Possibly these fungi can develop in plant tissues as endophytes causing damages only if suitable conditions appeared (weakness of plants).

The results demonstrated a possibility of the use of PCR-based diagnostics of infection at early stages of invasion of seedlings. It was also indicated that the mycorrhizal fungus *W. mikolae* did not cause the defense of damaged seedlings even though it was seldom and found in microflora of pine needles in trace quantities. In general, the analysis of roots identified 7 different fungus species, and two of them were also detected in infected needles as pathogens. Other micromycetes were represented by mycorrhizal genera *Suillus*, *Tuber* and *Tomentella* (Aucina *et al.* 2007).

The highest diversity of observed twelve species of fungi was detected in soil. Six of the species were represented in at least 10% of examined samples,. Most common was the mycorrhizal fungus *W. mikolae* found in 100% samples. Infection of soil was confirmed by finding large quantities of *C. herbarum* – (70% of samples) and *A. alternata* (40% of samples). *C. pinus* was detected in 20% of soil samples. Novel species of the genus *Cryptococcus*, isolated from dead needles of *P. sylvestris*, was identified using mycocinotyping and rDNA sequence data by Golubev *et al.* (2008). Phylogenetic analysis showed that the novel species was located in the *Kwoniella* clade of the Tremellales and was closely related to *Cryptococcus dejecticola*. The question whether the pathogen was introduced into the forest from a nursery is pending for the future research.

There is still an open question why the genera *Fusarium*, *Rhizoctonia* and *Trichoderma* were underrepresented in the study? DNA identification was based on universal primers applied for fungi (including *Fusarium* and *Alternaria*). Even if damping-off seedlings was caused by *Fusarium* this was not proved by DNA analyses which indicated that *Fusarium* was not present in the samples of needles and roots. It was rather the period of sampling or soil condition that were not appropriate for *Fusarium, Rhizoctonia* and *Trichoderma* development. During sampling in May, the weather was very dry and soil in the nursery was very poor in humus content (1–1.5%).

Conclusions

The development of new molecular technology seems to be essential for quick detection of pathogenic species in forest nurseries. In our study, the amplification of ribosomal ITS1–5.8S RNA-ITS2 region resulted in

identification of pathogenic DNA in 80% of infected seedlings.

Pathogenic fungal species were found in 57% of shrunken needles. The size of it's amplicon (with ITS1 and ITS4 primers) was 630 bp, and the sequence was deposited as GQ413953 in NCBI Genbank.

Observed in the forest nursery infection of seedling needles was caused mainly by three species of pathogenic fungi: *Cladosporium herbarum, unknown fungus* GQ413953 and *Alternaria alternata*. DNA of pathogens was detected in the roots of seedlings with no signs of needle shrinkage that would have showed the infection of plants.

Analysis of soil samples revealed presence of *C. herbarum, unknown fungus* GQ413953 and *A. alternata* which points to contamination of soil by phytopathogens.

DNA of mycorrhizal fungi was found in the roots of most seedlings. Most often found fungus was *Wilcoxina mikolae*, being also most common in soil. Other micromycetes were represented by the mycorrhizal genera *Suillus*, *Tuber*, and *Tomentella*.

Due to the fact that some sequenced DNA fragments were not found in the geen bank – NCBI we susspect the occurrence of new species – which is the subject for the future research.

Summing up, molecular genetic markers offer quick and reliable tool for identification of fungi causing infectious diseases of seedlings as well as detection of soil borne pathogens in forest nurseries. Such genetic diagnosis is crucial to implement right phytosanitary measures in order to limit development of pathogenic organisms in affected nurseries.

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